Mechanism of translational initiation in prokaryotes

IF3 is released from ribosomes during and not before 70 S initiation complex formation

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Received 30 September 1985; revised version received 21 November 1985

Abstract not supplied

Protein synthesis *Initiation factor* *Initiation factor 3 recycling* *Ribosome* N-*Ethylmaleimide*

1. INTRODUCTION

Prokaryotic initiation factors are not stably associated with ribosomes; after promoting the assembly of the initiation complex (70 S ribosomes-mRNA-fMet-tRNA), the factors dissociate from the ribosome before it enters the elongation cycle [1–3]. However, the mechanism and the timing of the ejection of the initiation factors still remain unclear. IF1 is released during subunit association, but it must have at least a transient association with the 70 S ribosome, so as to affect both the on- and off-rates of subunit association [2] and IF2 is released from the 70 S initiation complex upon GTP hydrolysis, even though the hydrolysis of GTP is probably not directly responsible for its ejection [4].

In the case of IF3, early experiments had suggested that the factor is displaced by the binding of the 50 S ribosomal subunits to the 30 S initiation complex [5,6]. Later reports, however, indicated that IF3 is released at an earlier stage, namely upon the binding of IF2 and fMet-tRNA to the 30 S ribosomal subunits [7–9], and this is the view currently endorsed by the majority of the published initiation schemes (e,g, [1,3]).

The picture of IF3 being released from 30 S ribosomal subunits upon binding of an IF2-fMet-tRNA complex, before the binding of a 50 S ribosomal subunit is very difficult to reconcile with our current view of the function of IF3 and also with some recent data obtained in vivo (see below).

The alternative pathways for the ejection of IF3 from the 30 S ribosomal subunit are shown in fig.1 where scheme 1 represents the ejection mechanism proposed by Van der Hofstad et al. [9], while scheme 2 represents an updated version of the ejection mechanism proposed originally [5,6].

Here we have reinvestigated the timing of IF3 release from ribosomes. This was done by exploiting the protection from *N*-ethylmaleimide (MalN-Et) modification conferred by 30 S ribosomal subunits on the single Cys SH group of IF3 [10]. The present data confirm the earlier findings that IF3 is released according to scheme 2 of fig.1, upon subunit association and not during the assembly of the 30 S initiation complex.

2. MATERIALS AND METHODS

GTP and bacteriophage R17 RNA were obtained from Boehringer Mannheim. *N*-[*ethyl*-2-^{3}H]Ethyl-

This paper is dedicated to Professor Mario Ageno on the occasion of his 70th birthday.

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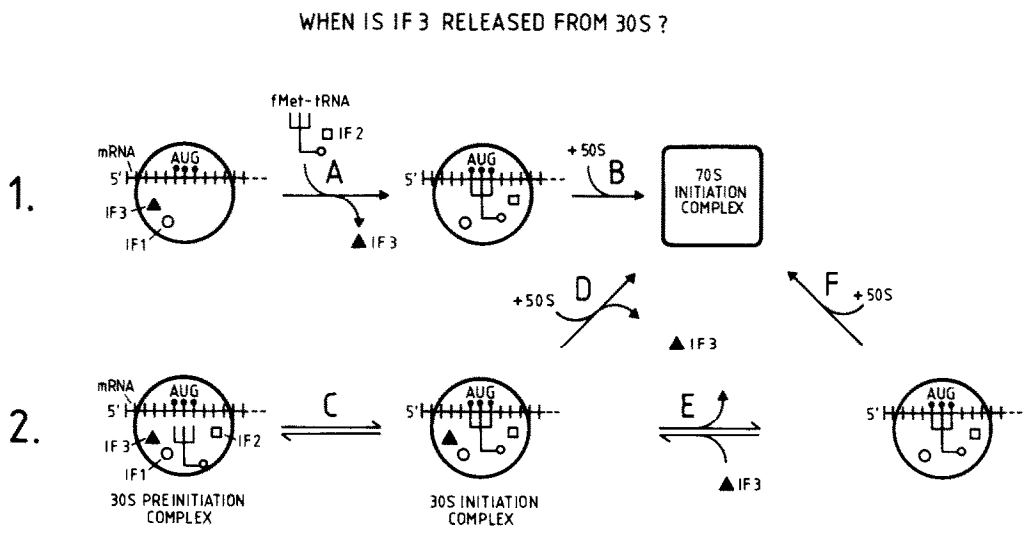

Fig.1. The alternative mechanisms of IF3 released from 30 S ribosomal subunits. Schemes 1 and 2 illustrate the alternative mechanisms proposed for the ejection of IF3 from 30 S ribosomal subunits. Both pathways lead to the formation of the same end product, i.e. the 70 S initiation complex. Scheme 1 begins with a 30 S–mRNA complex containing IF3 and IF1 (according to this model, the IF3-dependent binding of mRNA to the ribosomes is regarded as the first event in initiation). In step A, a binary complex of IF2 and fMet-tRNA interacts with the 30 S–mRNA complex to form a 30 S initiation complex. The ejection of IF3 occurs during step A since IF3 and the fMet-tRNA–IF2 complex are said to be mutually exclusive on the 30 S ribosomal subunits [9]. Scheme 2 begins with a pre-ternary complex, a 30 S ribosomal subunit with all 3 initiation factors bound to it and having both mRNA and fMet-tRNA sites occupied. Interaction between the initiation codon of mRNA and the anticodon of fMet-tRNA takes place in the subsequent first-order, rate-limiting ribosomal transition C producing the '30 S initiation complex' [18,20]. The 50 S ribosomal subunits associate with this complex and eject IF3 from the 30 S ribosomal subunits either directly (via step D) or indirectly by sequestering 30 S initiation complexes (step F) from which IF3 has fallen off. Even though formally different from the mechanism of scheme 1 the ejection of IF3 in step E would be practically equivalent to it if equilibrium E were shifted to the right even in the absence of 50 S ribosomal subunits.

maleimide (525 mCi/mmol) was purchased from New England Nuclear. All other chemicals were obtained from Merck (Darmstadt). *Escherichia coli* MRE 600 30 S and 50 S ribosomal subunits and purified initiation factors were obtained as described [11,12]. Formyl[^{3}H]Met-tRNA$_f^{Met}$ was prepared following the published procedure [13]. MalN-Et modification of Cys 66 of IF3 was carried out essentially as described in [10]; further details are given in the legend to fig.2.

3. RESULTS

Each 30 S ribosomal subunit binds one molecule of IF3 with a $K_a \simeq 2.5 \times 10^7$ M^{-1} [14]. Binding of IF3 to the large ribosomal subunits is negligible as long as they are in their native state and not perturbed structurally by being deproteinized [15] or unfolded [16]. The addition of 50 S ribosomal subunits to 30 S subunits bearing IF3 promotes the ejection of the factor [5,6]. These results were originally obtained by sucrose density gradient centrifugation and were recently reproduced by 'Airfuge' centrifugation ([2]; Pawlik et al., unpublished). The above results were interpreted to mean that the formation of the 70 S initiation complex is accompanied by the release of IF3. However, the earlier experiment still failed to rule out completely the alternative possibility that the ejection of IF3 might be caused by the binding of IF2 and fMet-tRNA to the 30 S ribosomal subunits since a small and variable amount of IF3 was lost from the 30 S ribosomal subunits upon formation of the 30 S initiation complex [5].

To resolve the discrepancy concerning the timing of IF3 ejection from ribsomes, we exploited the finding that the modification of the single Cys residue of IF3 (Cys 66) by MalN-Et is substantially reduced when the factor is bound to the 30 S ribosomal subunits [10]. Thus, we followed the time course of IF3 modification using radioactive MalN-Et under various experimental conditions; IF3 was labelled alone as well as in the presence of 30 S and of various combinations of the other initiation components. After the reaction, the incubation mixtures were subjected to electrophoretic separation and the amount of radioactivity associated with the IF3 was determined (fig.2). Panel A shows the time course of the reaction of IF3 with MalN-Et in the presence of either 30 S or both 30 S and 50 S ribosomal subunits. As seen in the figure, the rate of IF3 modification is higher in the presence of the 50 S subunits than in their absence. This increased reaction rate can be explained by the release of IF3 from its complex with the 30 S ribosomal subunit by the 30 S–50 S interaction, leading to the formation of the 70 S ribosome. Thus, a substantial amount of IF3, previously protected by the 30 S ribosomal subunit, now becomes available for modification.

In panel B, the rate of IF3 modification was measured as in panel A but in the presence of IF1, IF2 and GTP. The results are qualitatively similar to those in panel A; in this case, however, the difference between the samples reacted in the presence and absence of 50 S ribosomal subunits is even greater than before. This can be explained by an increase in the association constant of the 30 S-IF3 complex caused by IF1 and IF2 (Pawlik et al., unpublished) which results in a greater protection of IF3 against the MalN-Et reaction. Also, the rate of IF3 modification in the presence of 50 S subunits is somewhat reduced by the presence of the other 2 factors; the reduction however is less than that caused by 30 S, and can be explained by the greater difficulty encountered by the 50 S ribosomal subunit in ejecting IF3 from its more stable complex with the 30 S subunits and the other 2 factors.

Panel C shows an experiment in which the MalN-Et reaction of IF3 was followed in the presence of 30 S, with or without 50 S subunits, in the additional presence of IF1, IF2, GTP and either (i) fMet-tRNA$_f^{Met}$ (ii) R17 RNA or (iii) both fMet-tRNA$_f^{Met}$ and R17 RNA. These are the conditions for the formation of a complete 30 S or 70 S initiation complex.

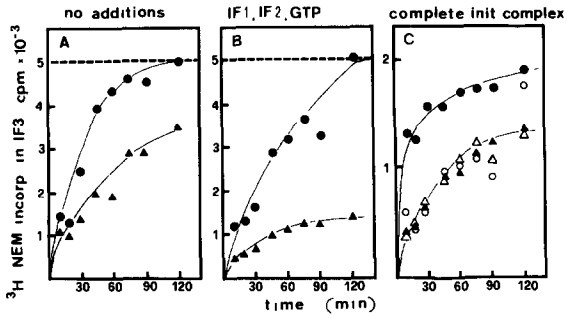

Fig.2. Time course of IF3 modification with MalN[^{3}H]Et. The reaction conditions and electrophoretic analysis of the products are essentially those previously described [10]. Before each experiment, the ribosomes and initiation factors were preincubated with dithiothreitol (DTT) and exhaustively dialyzed without DTT under nitrogen [10]. The reaction buffer contained 10 mM Tris-HCl, pH 7.8; 100 mM NH_4Cl; 7 mM Mg acetate. Each assay was carried out with 0.2 ml of buffer from which 20 μl samples were withdrawn at the indicated times, mixed with DTT (final concentration 20 mM) and subjected to electrophoresis on a 15% polyacrylamide gel containing SDS. The radioactivity associated with the band of IF3 was determined as described in [10]. The horizontal dashed line represents complete (100%) modification of Cys 66 of IF3. Each experimental point represents the labeling of 22 pmol IF3 in the presence of 22 pmol *E. coli* 30 S ribosomal subunits without (▲) and with (●) 22 pmol 50 S ribosomal subunits and with the following additions: A, none; B, 50 pmol IF1, 20 pmol IF2 and 1 mM GTP; C, same as in B with 20 pmol fMet-tRNA$_f^{Met}$ and 22 pmol R17 RNA – (○) R17 RNA omitted, (△) fMet-tRNA$_f^{Met}$ omitted. MalN[^{3}H]Et (525 mCi/mmol) was added to a final concentration of 0.3 mM after all the other components had been incubated for 10 min at 37°C and the MalN-Et modification was allowed to proceed at the same temperature for the indicated times. The percentage of 30 S ribosomal subunits active in 30 S initiation complex formation in the complete system was separately determined by millipore filtration and found to be approx. 40%.

Separate control experiments carried out with radioactive fMet-tRNA$_f^{Met}$ by nitrocellulose filtration (not shown) confirmed that these complexes were indeed formed. As seen in the figure with 30 S subunits in the presence of all the combinations of initiation components, the MalN-Et modification of IF3 is depressed to the same rate and ex-

tent as that seen in panel B. Only upon addition of 50 S ribosomal subunits does a substantial deprotection of IF3 take place; this shows that a large portion of the factor has been released from the 30 S ribosomal subunit during the transformation of the 30 S initiation complex into a 70 S initiation complex. The initial rate of modification of IF3 upon its release from 30 S ribosomal subunits is similar to that observed in panel B. After 30 min, however, the reaction seems to approach a plateau at a level corresponding to approximately 40% modification. This reduced yield can be explained by the fact that upon release from ribosomes, IF3 may bind to the excess R17 RNA present in the mixture, thereby becoming partially shielded.

4. DISCUSSION

We have shown here that IF3 is strongly protected from chemical modification by MalN-Et whenever it is bound to the 30 S ribosomal subunit, regardless of the presence of other initiation components, and that it becomes accessible for reaction only when 50 S subunits are added to the system. These results demonstrate that IF3 is not released from 30 S ribosomal subunits by the other 2 initiation factors, by mRNA, by fMet-tRNA or upon formation of the 30 S initiation complex, and that, if it indeed exists, equilibrium E in fig.1 is completely shifted to the left when no 50 S ribosomal subunits are present. The results indicate, on the other hand, that IF3 is ejected from the 30 S subunits by the 50 S ribosomal subunits upon formation of the 70 S initiation complex. These conclusions confirm the earlier data obtained by sucrose density gradient centrifugation [5,6], but are at variance with the currently accepted scheme (fig.1 scheme 1) derived from data from other laboratories [7–9]. Several reasons could account for this discrepancy.

(i) The use of a short form of IF3, missing the first 6 amino acids, by other groups (as documented in [17]) might be responsible for the apparent release of IF3 at an earlier stage, since such an IF3 form displays a substantially reduced affinity for the 30 S ribosomal subunit ([2] and Lammi et al., in preparation).

(ii) The observation of a release of IF3 before 70 S initiation complex formation depended on the fixation of the complexes with glutaraldehyde and it is common knowledge that this procedure can cause artefacts.

(iii) The formation of a small amount of 70 S initiation complex due to the presence of contaminating 50 S subunits in the 30 S ribosomal subunit preparations could give the misleading impression of an early release of IF3, as would also the use of an excessive amount of RNA (either tRNA or mRNA) which, being for the most part unattached to ribosomes, would draw away from the 30 S ribosomal subunits a portion of their bound IF3 [6].

Apart from the experiments presented here, the pathway of IF3 release according to scheme 1 of fig.1 can be ruled out by other considerations. IF3 affects both on- and off-rates of step C (fig.1, scheme 2) and therefore it is likely to be bound to the molecular species present on both sides of this equilibrium [18–20]. Furthermore, if the release of IF3 occurred according to scheme 1, excess free IF3 would probably inhibit translational initiation both in vivo and in vitro by preventing 30 S initiation complex formation and this is not the case (see below). Finally, it was found that the putative fMet-tRNA–IF2 complex, which according to scheme 1 is supposed to displace IF3 from ribosomes, is probably not an intermediate in 30 S initiation complex formation (Gualerzi and Wintermeyer, in preparation).

According to scheme 2 (fig.1), IF3 could be released in either 1 of 2 possible ways: either displaced directly by the 50 S subunits as in step D or indirectly by spontaneous dissociation according to equilibrium E. The latter is tantamount to the statement that the 50 S ribosomal subunits can only associate with a 30 S initiation complex depleted of IF3 (step F). Our experiments do not allow us to discriminate between these 2 mechanisms. It should be mentioned, however, that, upon site-directed mutagenesis of the initiation region of the *infC* gene in a multicopy plasmid, we have obtained the constitutive synthesis of very large amounts of IF3, corresponding to a several-fold excess of IF3 over ribosomes. Such an excess of IF3 would be expected to push equilibrium E over to the left and might thereby slow down the process of initiation. However, no altered phenotype was observed with these cells which had the same growth rate and reached the same cellular density as the wild type control. This evidence is

not conclusive, but we are inclined to favor the idea that IF3 is ejected by the direct action of the 50 S subunits. This mechanism would also account for the finding of a transient interaction of IF3 with the 70 S ribosomes by rapid-reaction techniques [21].

ACKNOWLEDGEMENT

We are very grateful to Dr P. Woolley for the many stimulating discussions and for critically reading the manuscript. Supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 9.

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